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IMMUNOLOGICAL APPROACH TO THE IDENTIFICATION AND DEVELOPMENT OF VACCINES TO VARIOUS TOXINS

ANNUAL REPORT

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Monoclonal antibodies specific for the sodium channel blockers saxitoxin (STX) and tetrodotoxin (TDT) with relatively high affinities of binding (TD2C5 anti-TDT and S1A5 and S83.2 anti-STX antibodies have been generated and characterized as to their abilities to protect against toxin-induced in vitro toxicity and to induce anti-idiotypic immune responses. In a continued effort to enhance the binding affinities of the anti-toxin monoclonal antibodies, two novel approaches have been undertaken. The first one involves the design of a new toxin conjugate for use in immunization of BALB/c mice. The second approach involves the in vitro stimulation with free STX or TDT of mouse spleen cells hyperimmunized in vivo. Monoclonal anti-TDT and anti-STX antibodies have been generated that are protective against TDT and STX toxicity, respectively, on nerve functions. Rabbit anti-idiotypic antibodies specific for TD2C5 anti-TDT have been generated, purified and used in immunization of mice in order to test the potential use of anti-idiotype as a vaccine in inducing a systemic anti-TDT immune response.

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Block 19. (continued)

A monoclonal anti-idiotypic antibody (DE8) specific for a protective anti-T-2 monoclonal antibody (HD_{11}) was generated and characterized. Administration of DE8 anti-idiotypic antibody in mice induced a significant anti-T-2 antibody response which was HD_{11}-positive and which could be inhibited by T-2-BSA, but not by free T-2. Mice have been immunized with the anti-idiotypic antibody DE8 conjugated to KLH and will be used to assess the ability of DE8 to induce a protective immune response against the in vivo T-2 toxicity.

Foreword

In conducting the research described in the report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources - National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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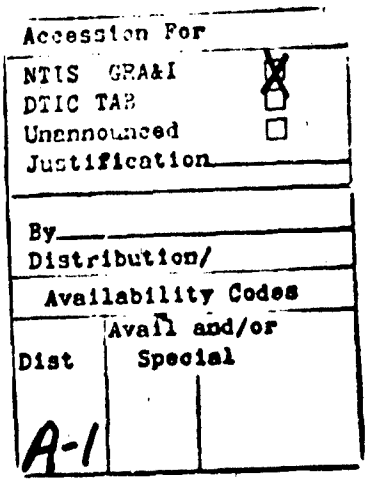

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A. Generation of Monoclonal Antibodies to Saxitoxin (STX)

In our continued attempt to generate monoclonal antibodies specific for STX with high binding affinities, two further fusions were performed with spleen cells from mice immunized with STX conjugated to keyhole limpet hemocyanin (KLH) using formaldehyde. A total of 1,799 hybrids were screened by STX-bovine serum albumin (BSA) ELISA (data not shown). A total of 21 (1.2%) hybrid supernatants were found to be positive for binding and were inhibited by free STX but not irrelevant antigens. However, none of these monoclonal antibodies exhibited relative binding affinities greater than 10^5 M^{-1}.

Two new approaches have been initiated in an attempt to increase the binding affinities. The first one involved an in vitro secondary immunization of STX-primed mouse spleen cells prior to fusion. Spleen cells from STX-KLH immunized mice were cultured in the presence of RPMI 1640 with 10% fetal bovine serum (FBS), 20% allogeneic supernatant and 1 uM STX. Allogeneic medium was prepared by co-culturing dissociated spleen cells from two histo-incompatible mice. Spleen cells from BALB/c and C57Bl at a density of 10^6 cells/ml were mixed at a ratio of 1:1 in 10% serum-containing RPMI 1640 medium for 3 days. The in vitro secondary immunization culture was carried out at a density of 5 x 10^6 cells/ml for 4 days. Cells were then harvested by centrifugation and fused to NS-1 mouse myeloma cells. The resulting hybrids were screened for binding to microtiter wells coated with STX-BSA formaldehyde conjugate. The percentage of positive anti-STX hybrids was considerably enhanced by the in vitro secondary immunization technique (Table 1), ranging from 12.3 to 20.6%. The increased percentages presumably reflect selection for anti-STX reactive spleen cells and their expansion during the secondary immunization culture period. Although a number of these clones demonstrated specific reactivity to STX and not tetrodotoxin (TDT), IC_{50} analysis of the antibody-containing supernatants of these cultures, to date, has not revealed any of these to have strong binding affinities to STX.

The second approach involved the development of a potentially highly immunogenic STX conjugate by coupling STX to KLH via the linker N-succinimidyl 3-2 (pyridyldithio) proprionate (SPDP). To prepare the conjugate, 50 mg of KLH in 15 ml of borate buffered saline (BBS, pH 7.25) was added to 5.2 mg of SPDP and 200 ul dimethyl formamide (1000:1 molar excess). The mixture was reacted for 1 hour at room temperature and dialyzed overnight against BBS, pH 8.0, at 4°C. To the KLH-SPDP was added 3.85 mg of dithiothreitol in BBS. The mixture was dialyzed against 2 l of BBS containing 3 mM sodium borohydride for 1 hr at 4°C. The buffer was changed and dialysis performed against BBS for another hour at 4°C. The STX derivative was then added to the dialysate. The derivative was prepared by drying 5 mg STX under vacuum followed by heating at 110°C for 3 hr in the presence of 7.5 N HCl. The resulting decarbamoyl-STX was dried under vacuum. The STX-SPDP-KLH conjugate was reacted for 2 hr at room temperature and dia-

lyzed overnight. The dialysis buffer was changed 2 times during the next 2 days. Binding ELISA assay indicated the presence of STX on the prepared conjugate although the exact amount present is not known. Three BALB/c mice were immunized with this newly prepared STX-KLH conjugate, and the anti-STX antibody titers following the quaternary injections are shown on Table 2. The mouse with the highest antibody titer was recently sacrificed and its spleen cells were fused for the generation of monoclonal anti-STX antibodies.

B. Generation of Monoclonal Antibodies Specific for Tetrodotoxin (TDT)

In addition to the relatively high binding monoclonal anti-TDT antibody (TD2C5), another clone, termed OB10, was isolated and was determined to have a K_a of 0.20×10^7 L/M and to be of the IgG1 subclass. Some of the characteristics of the OB10 clone are described below.

C. Protective Effects of Anti-STX and Anti-TDT Antibodies

1. Brain Receptor Assay. Using the brain receptor assay, the protective effect of anti-STX or anti-TDT antibodies could be evaluated. This brain receptor assay has been described in previous reports. For anti-STX antibodies, rat brain cells membranes were incubated with [^{3}H] STX under saturating conditions (6 nM, final concentration) in the presence or absence of antibody to STX for 1 hr at 4^{o}C. The difference in [^{3}H] STX binding in the presence and absence of anti-STX represented the amount of [^{3}H] STX bound by the antibody. The amount of [^{3}H] STX bound by antibody was then calculated from the specific activity of [^{3}H] STX. For anti-TDT antibodies, the brain receptor assay was performed by incubating anti-TDT monoclonal antibodies for 1 hr at 37^{o}C with various concentrations of STX or TDT in the presence of membranes and [^{3}H] STX. The binding curves generated in the presence of antibody were compared with those of toxin vs. [^{3}H] STX alone. The displacement or difference in toxin bound at 50% inhibition of [^{3}H] STX binding in the presence and absence of antibody yielded the amount of toxin bound by the antibody, as described in the first annual report. The results obtained with the brain receptor assay employing anti-TDT monoclonal antibody (TD2C5) were presented in the first annual report. Two monoclonal antibodies specific for STX (S1A5 and S3E.2) were tested for their ability to bind STX and prevent its binding to the receptor. S3E.2 (5.64 ug) was able to displace 0.89 ng (1.3 ng - 0.4 ng) of STX at the 50% inhibition point (Figure 1). In terms of molar capacity, 1 nmol of S3E.2 bound 0.09 nmol of STX. In comparison, S1A5 (1 nmol) was able to bind only 0.005 nmol STX (data not shown).

2. In Situ Protection of Peripheral Nerve Function by Anti-STX and Anti-TDT Antibodies. In order to test the in situ protective ability of TD2C5 antibody, thirty male Sprague-Dawley rats (age 40 to 60 days) were used in collaboration with Dr. Deborah Armstrong of the University of Texas, San Antonio.

Sodium Pentobarbital (Nembutal) was administered intra- peritoneally (60 mg/kg body weight) and the rats were placed on an isothermal heating pad. The left rear leg and hip were shaved and the leg was immobilized on a small surgical platform. A dissection was performed to expose the sciatic nerve and tibial branch entering the gastrocnemius muscle. The skin surrounding the incision was lifted and secured with hemostats to form an enclosure for bathing the nerve in warm 0.9% NaCl that contained the various antibody (TD2C5) and TDT concentrations. The solutions were applied and withdrawn using pasteur pipettes.

Bipolar stimulating and recording electrodes manufactured from Teflon insulated silver wire were positioned beneath the sciatic and tibial nerves, respectively. Single square wave pulses (0.05 msec duration) were used to evoke action potentials which were recorded with a WPI differential amplifier. The tibial nerve responses were displayed and photographed using a Tektronix storage oscilloscope. Response amplitude was measured as the difference between the most positive and negative deflection of the compound action potential waveform. The stimulus intensity (3 to 6 volts) was adjusted to produce maximal compound action potential amplitude. Prior to any application of antibody or toxin, the action potential amplitude was recorded every 30 sec for a 3 min period and the average of these six responses formed the baseline data for each preparation. Experimental treatment consisted of soaking the nerve for 5 min in either 5.55 uM TD2C5 antibody alone (control), 10 uM TDT alone, 10 uM TDT combined with 5.55 uM TD2C5 antibody (EXP 1), 10 uM TDT combined with 2.78 uM TD2C5 antibody (EXP 2), or 10 uM TDT combined with 0.55 uM TD2C5 antibody (EXP 3). When toxin and antibody treatments were carried out, the antibody was added to the toxin solution 1 hr prior to application. After 5 min of soaking the nerve, the solution was then removed and the average response amplitude was determined as described above for baseline data. The same solution was then re-applied for an additional 5 min, withdraw, and the average response amplitude was again determined. At the end of the experiment, the animal was sacrificed with an overdose of Nembutal.

Baseline and experimental responses were compared using analysis of variance (ANOVA) with a general linear model procedure (SAS Institute, Cary, NC). Data displaying an overall significance level of p less than 0.05 were further analyzed with the Neuman-Keuls multiple range test.

The results of the experiments are summarized in Table 3. When antibody was applied alone there was no significant change in response amplitude. However, application of the toxin alone reduced the compound action potential to 37% of baseline after 5 min and to 21% after an additional 5 min. The response was preserved in the presence of the two highest concentrations of antibody used, but at the lowest concentration of antibody the response was again significantly reduced in amplitude. Figure 2 provides examples of responses recorded following antibody alone (control), TDT alone, and TDT combined with 5.55 uM of the anti-

body (TD2C5). Based on these data as well as those from the brain receptor assay, TD2C5 thus protects against TDT-mediated toxicity.

In order to test the in situ protective ability of S3E.2 and S1A5 antibodies, the tibial nerve of the gastrocnemius muscles of male Sprague-Dawley rats (age 40 to 60 days) were exposed, to STX in the presence and absence of antibody. Nerve action potentials were recorded as described above. Baseline action potential amplitudes were measured prior to each experimental manipulation. STX (1 uM) with or without antibody was then added and the amplitudes recorded 5 and 10 minutes later. When toxin and antibody treatments were carried out, the antibody was added to the toxin solution 1 hr prior to application. As shown in Table 4, S3E.2 offered significant initial protection against STX at all concentrations tested even at 30-fold molar excess. Over time, the action potential decreased, although they remained greater than those with STX alone. Increasing the S3E.2 in the ranges shown had no import on this effect. This decline in anti-STX protection may reflect a re-establishment of equilibrium between STX binding to antibody ($K_a=10^6\ M^{-1}$) and STX binding to STX receptors on nerve and muscle ($K_a=10^9\ M^{-1}$). The wide disparity (1000-fold) in K_a between STX receptors and antibody binding affinity of S3E.2 could conceivably cause STX initially bound to S3E.2 to shift to the more strongly binding STX receptors. Such an effect may account for the observed gradual dampening of action potentials in the presence of STX and S3E.2. The S1A5 antibody was also submitted to similar testing in rat gastrocnemius muscle. Unlike S3E.2, it offered no protection against STX toxicity (data not shown). This is probably not surprising in view of the even lower K_a of this antibody for STX.

These data and those from the previous report suggest that S1A5, although useful for detecting STX by ELISA, would offer only modest protection against STX as judged by the brain receptor assay and by the in situ protection experiments. The S3E.2 antibody would appear to offer more protection against STX.

D. Anti-Idiotypic Antibodies against Anti-STX and Anti-TDT Antibodies.

1. Anti-TDT (TD2C5) Anti-Idiotypic Antibodies. A number of BALB/c mice have been immunized with TD2C5 conjugated to KLH in order to generate anti-idiotypic (anti-Id) antibodies. The levels of circulating anti-Id in immunized mice were assessed by a "sandwich" assay described in previous reports. Briefly, microtiter wells were coated with Ab-1 (TD2C5), incubated at 4°C overnight and blocked with PBS supplemented with 5% normal goat-serum (NGS). Serial dilutions of mouse sera were then added to the microtiter wells and incubated for 1 hr at 37°C. After washing off unbound antibodies, an appropriate dilution of TD2C5 conjugated to biotin (TD2C5-b) was added and reactivity was developed with the proper substrate. The anti-idiotype antibody titers of three representative mice immunized with TD2C5-KLH are presented in Table 5. A total of 4 fusions have been performed

with spleen cells from mice injected with TD2C5-KLH in order to generate monoclonal anti-idiotypic antibodies. Approximately 2000 resulting hybrids were tested for TD2C5 binding using the "sandwich" assay described above. Nine hybrid supernatants were found to be positive for TD2C5 binding (data not shown). The binding of the monoclonal anti-Id antibodies to TD2C5 appeared to be specific in that no significant binding was observed with monoclonal antibodies of other specificities (HD_{11} anti-T-2 and S1A5 anti-STX antibodies) or normal mouse Ig. However, the binding of the monoclonal anti-Id antibodies to TD2C5 did not appear to be at the antigen-binding site since neither free TDT nor TDT-BSA was able to inhibit this binding. These results suggest that the monoclonal anti-Id antibodies generated are specific for a site not associated with or distant from the antigen-binding site (Ab-2). Although anti-idiotypic antibodies which possess the "internal image" (Ab-2) represent ideal vaccine candidates, Ab-2 has been shown previously to be able to modulate immune responses. The characterization of these monoclonal anti-idiotypic antibodies specific for TD2C5 is under investigation.

Two rabbits were also immunized with unconjugated TD2C5 monoclonal antibody in alum. Serum from the third injection of rabbits immunized with alum-precipitated TD2C5 was extensively adsorbed against a column of normal mouse immunoglobulin (Ig) conjugated to Sepharose-4B to remove anti-isotypic and anti-allotypic antibodies. In order to prepare this column, 5 ml of normal mouse serum was salt cut with ammonium sulfate (50% final concentration), stirred for 30 min at 4°C and centrifuged for 20 min at 10,000 x g. The pellet was resuspended in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and the OD_{280nm} was determined. The total protein yield was 60 mg. Cyanogen bromide activated Sepharose-4B (4 mg) was suspended in 1 mM HCl to yield 14 ml of swollen gel and washed with 800 ml of 1 mM HCl on a glass filter. The 60 mg of Ig-containing protein was diluted to 20 ml in coupling buffer and mixed with the Sepharose overnight at 4°C on a rocker platform. Excess protein was washed off with 80 ml coupling buffer. Approximately, 10 mg of protein did not bind to the column. Any remaining active groups on the Sepharose-Ig gel were blocked with Tris-HCl buffer (0.1 M, pH 8.0) for 16 hr at 4°C on a rocker platform. The gel was then washed with three cycles of alternating pH: 100 ml of acetate buffer (0.1 M, pH4.0, 0.5 M NaCl) followed by 100 ml of Tris buffer (0.1 M, pH 8.0 with 0.5 M NaCl). Rabbit serum (10 ml) was then added to the Sepharose-Ig on a column and adsorbed overnight at 4°C on a rocker platform. The serum was eluted off and the column washed with borate-buffered saline (BBS) until OD_{280nm} read less than 0.01. Antibodies bound to the column (rabbit anti-mouse isotype and allotype antibodies) were eluted with 0.1 M glycine, pH 3.0 buffer until OD_{280nm} was less than 0.01 and the protein-containing fractions were saved. The column was then washed with 200 ml of BBS. Aliquots of the adsorbed serum and the column-bound antibodies were tested by ELISA for binding specificity.

ELISA plates were coated with 10 ug/ml of TD2C5 or 50 ug/ml

of a control mouse anti-T-2 IgG_1 antibody (HD_{11}) and 5% normal goat serum (50 ul/well) and incubated overnight at 4°C. The plates were blocked for 1 hr at 37°C with 5% normal goat serum (200 ul/well). Serially diluted (twofold) adsorbed rabbit serum (100 ul/well) were then added for 1 hr at 37°C. The plate was washed with PBS-Tween three times and 50 ul of goat anti-rabbit Ig conjugated to HRP was then added for 1 hr at 37°C. The plate was again washed and developed with ABTS. The serum was sufficiently adsorbed when binding was noted on the TD2C5 wells but not the HD_{11} wells. The ELISA from the final adsorption is shown in Table 6 and clearly demonstrates the specificity of the adsorbed serum for TD2C5 and not HD_{11}.

To further assess the specificity of rabbit anti-TD2C5 binding, the ability of rabbit anti-TD2C5 serum to prevent TD2C5 -b binding to TDT-BSA coated plates was tested by an inhibition assay. TDT-BSA coated plates were blocked with 5% normal goat serum in PBS for 1 hr at 37°C. TD2C5-biotin was diluted to 1:800 (50% binding) in BBS buffer. Dilutions of normal rabbit serum or anti-TD2C5 serum were mixed with an equivalent volume of TD2C5-biotin for 1 hr at 37°C prior to adding 100 ul of each mixture to the blocked ELISA plate for another hour at 37°C. The plate was washed and 50 ul of strep-avidin HRP at a 1:1000 dilution was added per well for 30 minutes at room temperature. The plate was washed and developed with ABTS. The rabbit anti-TD2C5 inhibited the binding of TD2C5-biotin in a dose-dependent fashion (Fig. 3). The normal rabbit serum had no effect on binding. These data suggest that at least one species of anti-TD2C5 is present in the serum that is specific for the binding site of TD2C5.

To further characterize the rabbit anti-Id response, free TDT was assayed for its ability to inhibit the binding of the rabbit anti-TD2C5 serum to TD2C5-coated plates. Various concentrations of TDT (0.2 to 200 ug/ml) were added to microtiter wells pre-coated with TD2C5 (10 ug/ml). The plates were incubated for 1 hr at 37°C and washed to remove unbound TDT. A 1:2500 dilution of anti-TD2C5 serum (50 ul) was then added for an hour at 37°C and the plate washed. Goat anti-rabbit IgG-horseradish peroxidase was added for another hour at 37°C. The plate was washed and developed with ABTS. Free TDT at any concentration was unable to inhibit the binding of rabbit anti-TD2C5 (data not shown). These results suggest that the rabbit anti-TD2C5 is similar to the rabbit anti-HD_{11} anti-idiotype (anti-T_2 toxin) described in a previous report, in that the anti-TD2C5 apparently possesses at least 2 species of anti-Id antibodies: 1) those specific for the TD2C5 binding site which inhibit binding of TD2C5 to TDT-BSA coated plates and 2) those recognizing non-antigen binding site Id determinants which are responsible for binding to TD2C5-coated plates in the presence of excess TDT.

The rabbit anti-TD2C5 has been purified further by affinity adsorption on a TD2C5-Sepharose column prepared as described for normal mouse serum Sepharose above. The anti-TD2C5 serum was adsorbed once on the column and most, if not all, of the anti-

TD2C5 was successfully bound to the column (data not shown). The adsorbed serum and the eluted antibody were tested for anti-TD2C5 reactivity using TD2C5 solid phase ELISA. This affinity purified rabbit anti-TD2C5 preparation will be used to immunize mice for the induction of anti-TDT antibody response.

Monoclonal anti-STX S1A5 was also conjugated to KLH and injected into BALB/c mice for the production of anti-idiotypic antibodies. Thus far, no circulating anti-Id antibodies specific for S1A5 have been detected in the immunized mice.

E. Induction of Anti-T-2 Antibodies in BALB/c Mice by Injection of Rabbit Anti-Id Antibody to HD_{11}

Employing HD_{11} alum precipitate as an immunogen, we have generated rabbit anti-Id antibodies that appear to recognize at least two specificities. One of these specificities is hapten-inhibitable, whereas the other is framework-associated and non-hapten inhibitable (for details, see the first yearly report).

To determine if the rabbit anti-Id antibodies could induce the production of an anti-T-2 immune response, four groups of five BALB/c mice each received the following antigen preparations:

1. T-2 conjugated to ovalbumin.
2. Rabbit anti-Id precipitated in alum.
3. Rabbit anti-Id conjugated to KLH and alum precipitated.
4. Rabbit anti-Id specific for an antibody to hepatitis B surface antigen prepared as in 3. This served as the negative control.

All mice receive bi-weekly injections. The first injection was performed in Freund's complete adjuvant followed by a boost in Freund's incomplete adjuvant. Both injections were done intradermally. Subsequent boosts were done with antigens in saline intraperitoneally. Mice were bled after the 4th injection and their sera assayed for the presence of anti-T-2 antibodies and for the expression of the original idiotype (HD_{11} or Ab1). The results are shown in Figure 4. The group of mice immunized with T-2-OVA gave a good anti-T-2 antibody response. Mice immunized with rabbit anti-Id conjugated to KLH also gave a significant anti-T-2 immune response, whereas unconjugated rabbit anti-Id induced a lower anti-T-2 antibody response. Mice immunized with the control anti-Id did not induce a detectable anti-T-2 response (data not shown).

The expression of the original Id on HD_{11} anti-T-2 monoclonal antibody by the anti-T-2 antibody response in these mice was also assessed. The results are presented in Figure 5. In this assay, microtiter wells were coated with 50 ug/ml of T-2-BSA and reacted with varying dilutions of mouse sera. After extensive washing, rabbit anti-HD_{11} was added, followed by goat anti-rabbit Ig conjugated to biotin, and reactivity was detected with

avidin-HRP and the appropriate substrate. The results showed that the anti-T-2 antibody response induced by the rabbit anti-Id either conjugated to KLH or unconjugated expressed a high degree of HD_{11} Id. On the other hand, the T-2-OVA-induced anti-T-2 response was devoid of detectable HD_{11} Id.

Taken together, these results showed that rabbit anti-idiotypic antibodies specific for HD_{11} anti-T-2 antibody could induce in naive mice the generation of an anti-T-2 antibody response. Although the anti-T-2 immune response induced by the rabbit anti-Id was of a lower magnitude compared to that induced by the native antigen (T-2-OVA), it consisted of a strong expression of Hd_{11} Id-positive anti-T-2 antibodies which have been shown to be protective against the *in vitro* cytotoxicity of T-2. As we have mentioned earlier, HD_{11} represents one antibody which possessed protective capability among approximately 100 monoclonal anti-T-2 antibodies derived from fusions of spleen cells from mice immunized with T-2-OVA. The frequency of protective antibodies from immunization with the native antigen thus appeared to be very low. Therefore, it is not unreasonable to speculate that by modulating the anti-T-2 antibody response toward the expression of protective antibodies such as HD_{11} as has been achieved with the rabbit anti-id, one may induce a more effective *in vivo* protection against T-2 toxicity.

F. Mouse Monoclonal Anti-Id Antibody Specific for HD_{11} Anti-T-2 Antibody

To generate monoclonal anti-Id antibodies against HD_{11}, a group of five BALB/c mice were immunized with 50 ug each of HD_{11} conjugated to KLH and alum precipitated. After four injections, the mice were sacrificed and their spleen cells fused with the NS-1 myeloma cell line. Supernatants obtained from wells with hybrid growth were screened using the following ELISAs. Microtiter wells were pre-coated with 50 ul of 10 ug/ml purified solution of HD_{11}. Nonspecific sites were blocked with PBS supplemented with 5% normal goat serum. Culture supernatants were then added to the wells and incubated for 1 hr at 37°C. After washing away unbound antibodies, reactivity was developed with HD_{11} conjugated to biotin (HD_{11}-b) and the appropriate substrate. This assay is designed to assess the ability of the culture supernatants to bind to HD_{11}. A second ELISA was also performed concurrently. In this assay, microtiter wells were coated with 50 ug/ml of T-2-BSA. Mixtures of a concentration of HD_{11}-b which gave approximately 50% T-2 binding and culture supernatants were added to the wells. Reactivity was developed by addition of avidin-horseradish peroxidase (A-HRP) and the appropriate substrate. This ELISA is designed to test for the ability of hybrid supernatants to inhibit the binding of HD_{11} to T-2. Among approximately 800 hybrids tested, one was found to bind HD_{11} and to inhibit the binding of HD_{11} to T-2-BSA coated wells. This hybrid was subcloned by limiting dilution until more than 95% of the clones were reactive. One subclone, designated DE8, was chosen for further characterization.

1. DE8 Isotype. The isotype of the monoclonal antibody produced by DE3 hybrid cells was determined using two methods. The first method involves the direct staining of DE8 hybrid cells with goat anti-mouse isotype specific antibodies conjugated to FITC. The second method uses an isotyping ELISA kit (Biorad). Data obtained from both methods indicated that DE8 is an IgG_{2b} monoclonal antibody.

2. Specificity of the Binding of DE8 to HD_{11}. DE8 hybrid cells were injected into primed BALB/c mice to generate ascites. DE8 monoclonal IgG_{2b} antibody was purified from the ascites by caprylic acid precipitation as described in previous reports. To assess the specificity of the binding of DE8 to HD_{11}, binding and inhibition ELISAs were employed (Figures 6 and 7, respectively). In the binding assay, various concentrations of purified DE8 antibody were added to HD_{11} pre-coated wells. Binding activity was detected by the addition of HD_{11}-b and the appropriate substrate. As shown in Figure 6, the binding of DE8 to HD_{11} was dose-dependent. The 50% binding end point was obtained at approximately 7.8 ng/ml of DE8. Normal mouse serum and a control monoclonal antibody (S3E.2) did not show any significant binding to HD_{11} (data not shown). To further assess the specificity of binding, an inhibition assay was used. In this assay, various concentrations of DE8 were mixed with a constant concentration of HD_{11}-b which gave 50% binding and were added to wells pre-coated with T-2-BSA. For negative controls, normal mouse serum and control monoclonal antibody were used instead of DE8. Figure 7 shows that DE8 was very efficient in inhibiting the binding HD_{11}-b to T-2-BSA. As little as 3.6 ng/ml of DE8 inhibited HD_{11}-b binding to T-2-BSA by approximately 45%. On the other hand, normal mouse serum or control antibody did not inhibit the binding (data not shown).

3. DE8 Recognizes a Hapten-Inhibitable Idiotypic Determinant. To determine whether the idiotypic determinant recognized by DE8 is hapten-inhibitable, the following two assays were done. In the first assay, various concentrations of free T-2 were used to inhibit the binding of HD_{11}-b to DE8-coated wells. The data are shown in Figure 8. T-2 specifically inhibited the binding in a dose-dependent fashion. The 50% inhibition end point was obtained with approximately 10 ng/ml of free T-2. Saxitoxin and tetrodotoxin at concentrations as high as 200 ug/ml did not show any significant inhibition. In the second ELISA, various concentrations of free T-2 were used to inhibit the binding of DE8-b to HD_{11}-coated wells. The results are shown in Figure 9. Similar to data obtained in Figure 8, T-2 was efficient in inhibiting the binding of DE8-b to HD_{11}. Again, there was no significant inhibition with saxitoxin or tetrodotoxin at concentrations as high as 200 ug/ml (data not shown). Taken together, these results suggest that DE8 recognizes an idiotypic determinant which is specific for HD_{11} and which can be inhibited by free hapten T-2.

4. Rabbit Anti-Idiotype Inhibits DE8 Binding to HD_{11}. As mentioned above, we have generated a rabbit anti-idiotypic anti-

body specific for HD_{11}. This rabbit anti-idiotype recognized at least two different idiotypic specificities on HD_{11}. One of the specificities was shown to be associated with HD_{11}-binding site, whereas the other specificity was not inhibitable by T-2 and, therefore, must be associated with non-binding site idiotypic determinants. In order to assess whether the hapten inhibitable idiotypic determinant on HD_{11} recognized by rabbit anti-idiotype was the same as that recognized by DE8, we tested the ability of the rabbit anti-idiotype to inhibit the binding of DE8 to HD_{11}. In this assay, mixtures of various concentrations of the rabbit anti-idiotype and a constant amount of DE8-b which gave approximately 50% binding were added to wells pre-coated with HD_{11}. The results are shown in Figure 10. The data indicated that the rabbit anti-idiotype was efficient in inhibiting the binding of DE8-b to HD_{11}, whereas normal rabbit serum did not show any significant inhibition. The results suggest that the rabbit anti-idiotype and DE8 competed for similar T-2 binding sites on HD_{11}.

5. DE8 Monoclonal Antibody is not Cytotoxic for Hep-2 Cells. Since DE8 monoclonal anti-idiotypic antibody appears to carry the internal image of T-2, we tested the possibility that DE8 itself would mimic T-2 and thus would be cytotoxic to the human cell line Hep-2. The results indicated that DE8 itself was not cytotoxic to the Hep-2 cell line (data not shown). The lack of cytotoxicity may be due to the relatively large size of the DE8 antibody molecule as compared to T-2.

G. Induction of Circulating Anti-T-2 Antibody in BALB/c Mice by Injection of DE8 Antibody.

To assess the ability of DE8 anti-Id antibody to induce the formation of a circulating anti-T-2 immune response, 3 groups of 5 BALB/c mice each received the following immunizations:

1. T-2-ovalbumin
2. Unconjugated DE8 anti-Id precipitated in alum
3. DE8-KLH conjugate precipitated in alum

The first injection was performed intradermally in Freund's complete adjuvant, followed by a boost in Freund's incomplete adjuvant. Subsequent boosts were done with antigens in saline intraperitoneally. All mice received bi-weekly injections. The immunized mice were bled subsequently and the sera assayed for anti-T-2 antibodies and for the expression of the idiotype on the original Ab-1 preparation (HD_{11}). Figure 11 shows the anti-T-2 reactivity of the sera of mice after the third injection of T-2-OVA, DE8-alum or DE8-KLH-alum, as assessed by an ELISA employing T-2-BSA-coated microtiter wells. The results show that all 3 groups of mice made significant anti-T-2 antibodies following the third injection of antigens. It is interesting to note that the group of mice that received DE8-KLH-alum produced the highest titer of anti-T-2 antibodies, followed by the mice immunized with DE8-alum and T-2-OVA. These results confirm our previous observations that conjugation of an Ab-1 to a protein carrier such as

KLH induces a significantly higher immune response in a syngeneic system than unconjugated Ab-1 antibody. In order to assess the level of expression of HD_{11}^+ Id anti-T-2 in the 3 groups of mice, the following ELISA was utilized. Microtiter wells were coated with purified HD_{11} anti-T-2 antibody overnight at 4°C. After blocking of non-specific sites, mixtures of a dilution of DE8-biotin giving approximately 50% binding to HD_{11} with various dilutions of mouse sera were added to the microtiter wells and incubated for 1 hr at 37°C. After washing off unbound antibodies, DE8-b bound to HD_{11}-coated wells were detected by the appropriate substrate. The presence of HD_{11}^+ (HD_{11}-like) anti-T-2 antibodies in the mouse sera will inhibit the binding of DE8-b to HD_{11}-coated wells. The results are shown in Figures 12 and 13 for DE8-KLH-alum and DE8-alum immunized mice, respectively. Although the difference is not significant, it generally appears that DE8-KLH-alum immunized mice produced a somewhat higher level of HD_{11}^+ anti-T-2 antibodies. On the other hand, mice immunized with T-2-OVA did not produce significant levels of HD_{11}^+-Id antibodies. The range of inhibition of the binding of DE8-b to HD_{11}-coated wells by sera from mice immunized with T-2-OVA at 1:10 dilution ranged from 0 to 19.3% (data not shown). Thus, these data clearly demonstrated the ability to modulate an immune response by anti-Id antibodies to contain a particular Id^+-antibody, in this case HD_{11} anti-T-2 antibody, which we have shown earlier to protect against T-2 <u>in vitro</u> cytotoxicity.

To assess whether the anti-T-2 antibodies in the DE8-KLH-alum, DE8-alum, or T-2-OVA immunized mice could protect against Hep-2 cytotoxicity induced by T-2 toxins, various dilutions of mouse sera were added to a known concentration of T-2 toxin giving approximately 50% inhibition of [^{3}H]-leucine and incubated for 2 hrs at 37°C. The mixtures were then added to 96-well tissue culture plates containing monolayers of Hep-2 cells, followed by the addition of [^{3}H]-leucine and the incorporation of the radioactive amino acid was determined by scintillation counting as described in previous reports. The results in Figure 14 indicated that sera from mice immunized with DE8-KLH-alum or DE8-alum were effective in protecting Hep-2 cells against T-2 toxin-induced cytotoxicity, whereas sera from mice immunized with T-2-OVA were not as effective in protecting against T-2 cytotoxicity. The specificity of the anti-T-2 antibody response in all three groups of mice was demonstrated by the fact that their binding to T-2 was completely inhibited by 50 ul of a solution of T-2-BSA at 100 ug/ml (data not shown). BSA itself did not inhibit this binding. The titration of the inhibition by T-2-BSA is currently in progress. Interestingly, free T-2 toxin was not capable of inhibiting the binding of the anti-T-2 mouse sera to T-2-BSA.

H. <u>Future Plans</u>

We are continuing to employ the two novel approaches mentioned above in our attempt to generate anti-STX antibodies of high binding affinities. It is anticipated that the use of STX-SPDP-KLH conjugate as an immunogen will enhance our ability to

derive high binding monoclonal antibodies. The in vitro secondary stimulation of hyperimmunized spleen cells will also increase the likelihood of obtaining monoclonal anti-STX antibodies with high binding affinities.

We will concentrate our effort in generating monoclonal anti-idiotypic antibodies specific for TD2C5 anti-TDT and S1A5 anti-STX antibodies. Monoclonal anti-Id antibodies generated will be characterized and their potentials in inducing a systemic anti-toxin immunity will be assessed in mice.

A group of approximately 100 BALB/c mice are currently being immunized with DE8-KLH precipitated in alum in order to induce a systemic anti-T-2 antibody response. After approximately 4 to 5 injections and the demonstration of a significant circulating anti-T-2 immune response, these mice will be infected with T-2 toxin in order to assess the potential vaccine use of the anti-Id DE8 antibody.

TABLE 1

In-Vitro Secondary Stimulation

Fusions	No. of Hybrids Tested	No. of Positive Hybrids	% Positive
1	98	12	12.3
2	105	9	8.9
3	67	14	20.6

Spleen cells from mice hyperimmunized with STX-KLH were cultured in vitro with 1 uM STX as described in text and fused.

TABLE 2

Reactivity of Sera from Mice Immunized with STX-SPDP-KLH

Serum Dilution^{-1}	BALB/c Mice					
	A		B		C	
	P.I.[a]	Immune[b]	P.I.	Immune	P.I.	Immune
20	0.06	0.86	0.03	0.64	0.01	0.50
80	0.06	0.82	0.05	0.61	0.05	0.44
360	0.03	0.69	0.06	0.49	0.05	0.38

Mouse sera were assayed for binding to STX-BSA-coated microtiter wells.

[a] Pre-immune sera
[b] One week after the 4th injection

TABLE 3

Comparison of baseline and experimental nerve response amplitudes following treatment with 5.55 uM antibody alone (control), 10 uM TDT alone (TDT), 10 uM TDT combined with 5.55 uM antibody (EXP 1), 10 uM TDT combined with 2.78 uM antibody (EXP 2), and 10 uM TDT combined with 0.55 uM antibody (EXP 3)

Treatment N=6 per Group	Baseline	5 min	10 min
Control	1.29 ± 0.23	1.20 ± 0.25	1.10 ± 0.18
TDT	1.31 ± 0.23	0.49 ± 0.12*	0.29 ± 0.07*
EXP1	1.07 ± 0.08	0.96 ± 0.13	0.85 ± 0.08
EXP2	1.27 ± 0.10	1.06 ± 0.13	0.79 ± 0.20
EXP3	1.26 ± 0.08	0.69 ± 0.13*	0.40 ± 0.09*

*Significantly different from baseline response, $p < 0.05$, Neuman-Keuls.

TABLE 4

Comparison of baseline and experimental responses amplitudes of tibial nerve from rat gastrocnemius muscle in the presence or absence of STX and/or S3E.2 anti-STX antibody[a].

Treatment	Action Potential (mV)		
	Baseline	5 min	10 min
Control[b]	1.53 ± 0.19	1.48 ± 0.22	1.32 ± 0.20
STX[c]	1.43 ± 0.13	0.54 ± 0.14	0.13 ± 0.20
EXP1[d]	1.49 ± 0.21	0.91 ± 0.25	0.55 ± 0.09
EXP2[e]	1.50 ± 0.13	1.07 ± 0.06	0.65 ± 0.03
EXP3[f]	1.27 ± 0.18	0.68 ± 0.10	0.44 ± 0.09

[a] Data are the compound action potential amplitude in mV ± the S.E.M.
[b] n=4
[c] STX (1 uM), n=4
[d] STX (1 uM) and S3E.2 (5 uM), n=4
[e] STX (1 uM) and S3E.2 (10 uM), n=5
[f] STX (1 uM) and S3E.2 (30 uM), n=3

TABLE 5

Titration of Mouse Anti-TD2C5 Sera by Sandwich ELISA[a]

Mouse Serum Dilution^{-1}	Normal Serum	Anti-TD2C5 Sera		
		Serum 1	Serum 2	Serum 3
10	0.039	0.623	0.604	0.538
20	0.048	0.406	0.324	0.370
40	0.057	0.263	0.156	0.217
80	0.048	0.123	0.093	0.123
160	0.070	0.086	0.090	0.086
320	-	0.084	0.069	0.077
640	-	0.068	0.081	0.060
1280	-	0.068	0.071	0.057

[a] Microtiter wells were coated with 50 ul of 10 ug/ml TD2C5 as the solid phase. Mice received 6 biweekly injections with TD2C5-KLH.

TABLE 6

Specificity of Adsorbed Rabbit Anti-TD2C5 Serum

Rabbit Serum Dilution^{-1}	Microtiter Wells Coated with		
	Normal Mouse Ig	HD_{11}	TD2C5
640	0.05	0.08	>2.0
1180	0.06	0.05	>2.0
2560	0.04	0.04	>2.0
20480	0.05	0.04	>0.82

FIGURE LEGENDS

Figure 1. Competitive displacement of [^{3}H]STX binding to rat brain membranes by S3E.2. [^{3}H] binding was performed in the presence of STX alone (o) or STX plus 5.64 ug of S3E.2 (o) for 1 hr at 4^{o}C. The broken line represents the 50% inhibition of binding in the presence of S3E.2.

Figure 2. Comparison of baseline and experimental nerve response amplitudes following treatment with 5.55 uM TD2C5 antibody alone (top row), 10 uM TDT alone (middle row), or 5.55 uM TD2C5 plus 10 uM TDT (bottom row). Amplitudes were measured just prior to addition of the above compounds (baseline), then at 5 and 10 min post addition.

Figure 3. Inhibition of TD2C5-b binding to TDT by rabbit anti-idiotype ( ). Normal rabbit (x).

Figure 4. Rabbit anti-idiotype induces anti-T-2 immune response. Reactivity of mouse sera was detected in an ELISA with T-2-BSA coated plates.

Figure 5. Rabbit anti-idiotype induces an idiotype-positive T-2 response. The assay employed is described in the text.

Figure 6. Binding of HD_{11}-b to DE8-coated wells.

Figure 7. Inhibition of HD_{11}-b binding to T-2-BSA by DE8.

Figure 8. T-2 toxin inhibits the binding of HD_{11}-b to DE8-coated wells.

Figure 9. T-2 toxin inhibits the binding of DE8-b to HD_{11}-coated wells.

Figure 10. Rabbit anti-HD_{11} idiotype inhibits the binding of DE8-b to HD_{11}-coated wells.

Figure 11. Anti-T-2 reactivity of sera from mice immunized with DE8-KLH (o), DE8 ( ) or T-2-OVA ( ). Representative of results from single mouse serum in groups of 5 mice are shown.

FIGURE LEGENDS
(continued)

Figure 12. Presence of HD_{11}^{+}-Id anti-T-2 antibodies in sera of mice immunized with DE8-KLH. Results from all 5 mice are shown.

Figure 13. Presence of HD_{11}^{+}-Id anti-T-2 antibodies in sera of mice immunized with DE8. Results from all 5 mice are shown.

Figure 14. DE8 abolishes the protective effect of HD_{11} against T-2 toxicity of the Hep-2 cell line; DE8-KLH (x), DE8 ( ) and T-2-OVA ( ).

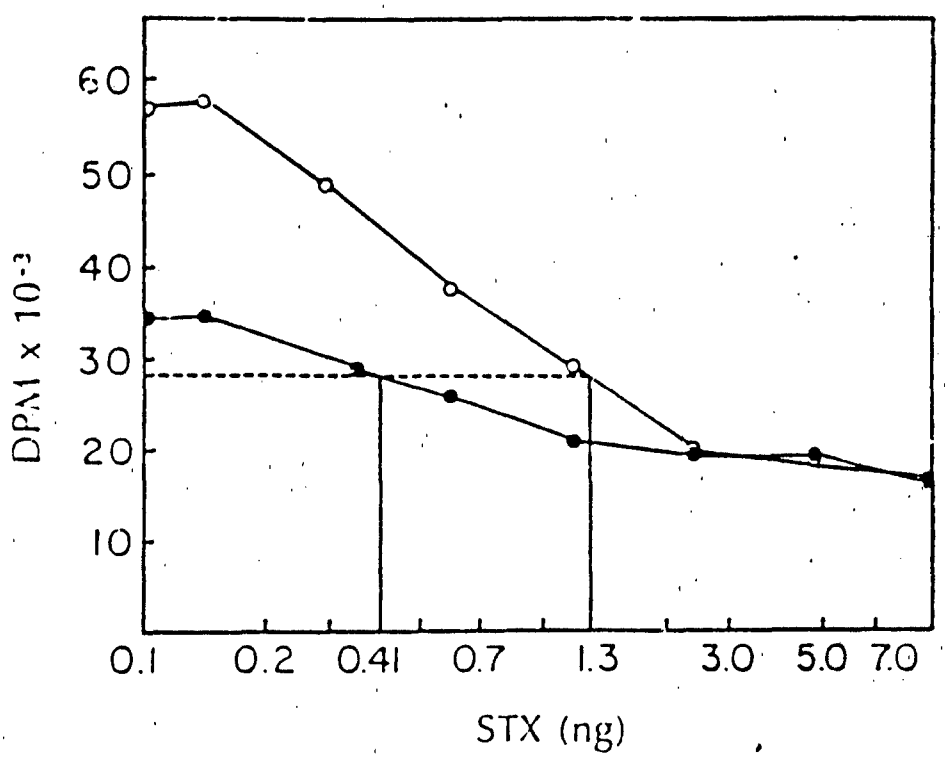
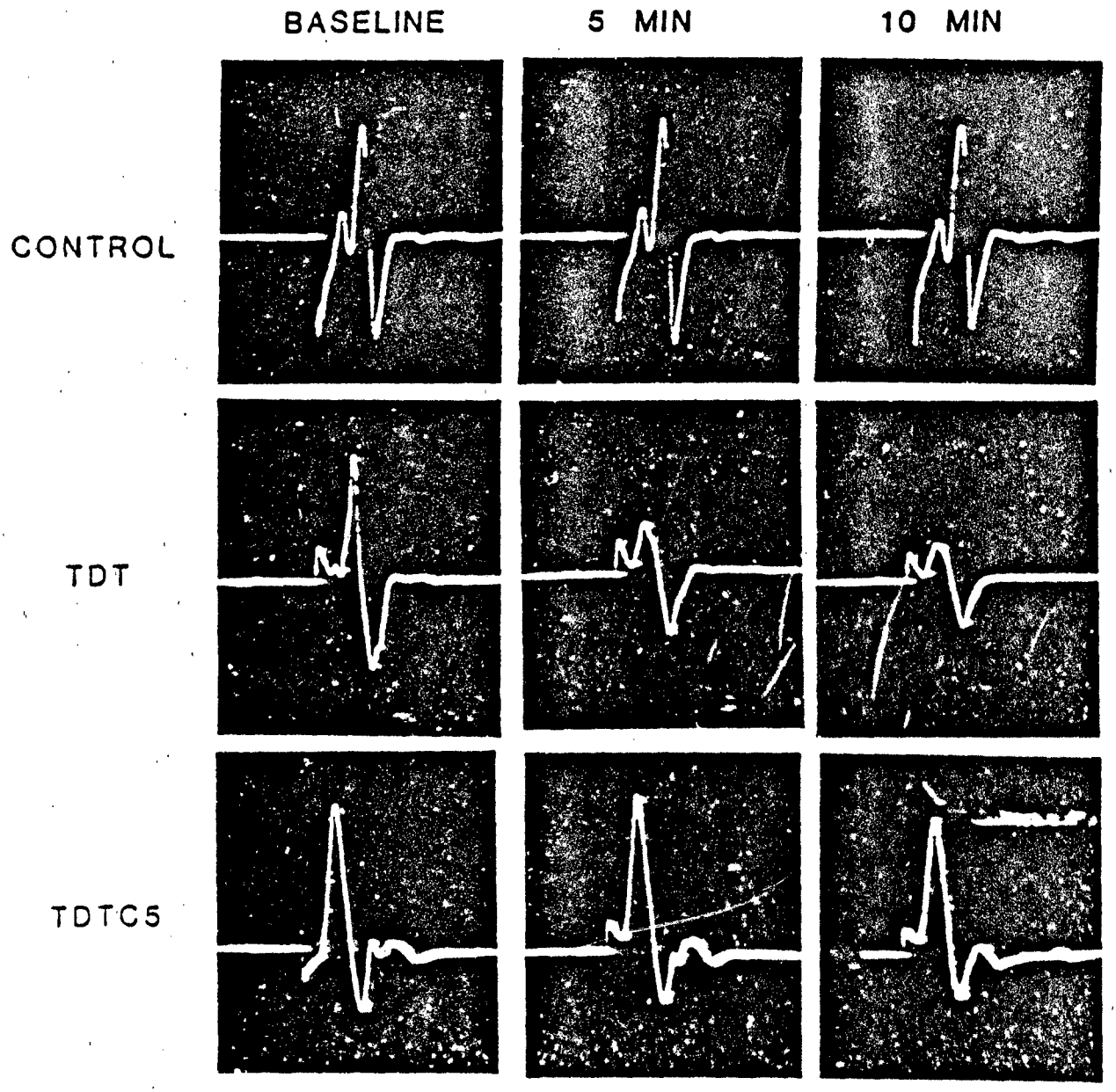


FIGURE 1



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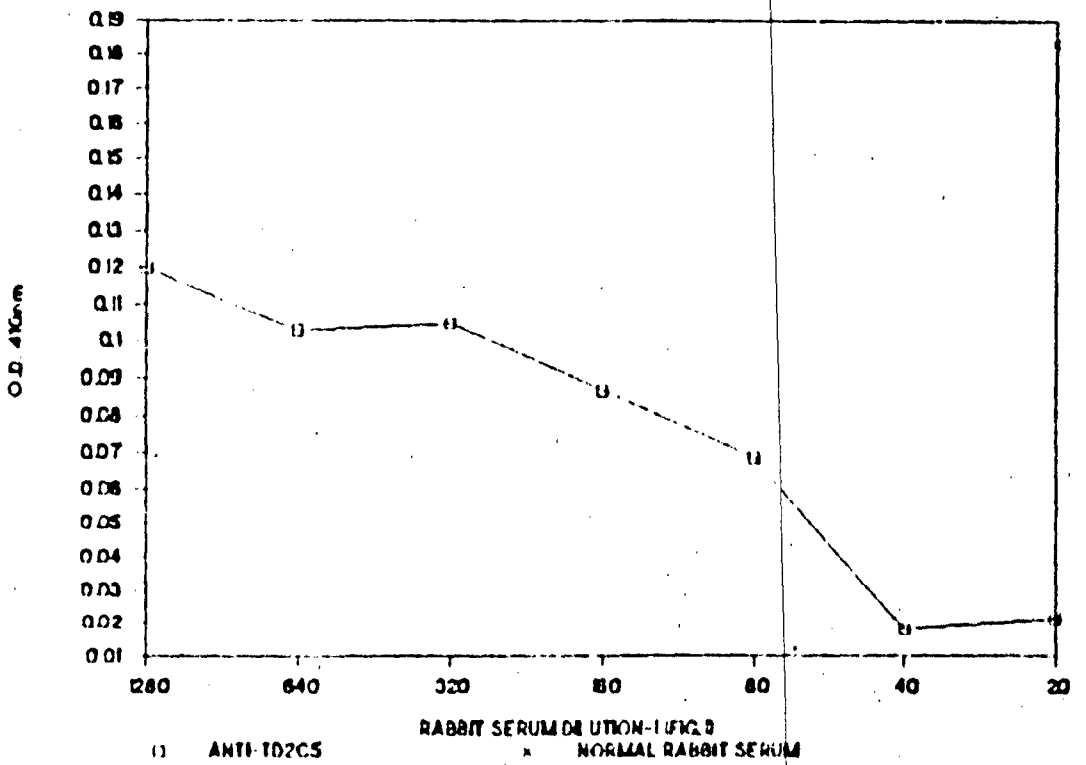
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0.07
0.06
0.05
0.04
0.03
0.02
0.01
O.D. 410nm
1280
640
320
160
80
40
20
RABBIT SERUM DILUTION-1/FOLD
ANTI-TD2C5
NORMAL RABBIT SERUM

FIGURE 3

Rabbit Anti-Idiotype Induces Anti-T-2 Immune Response

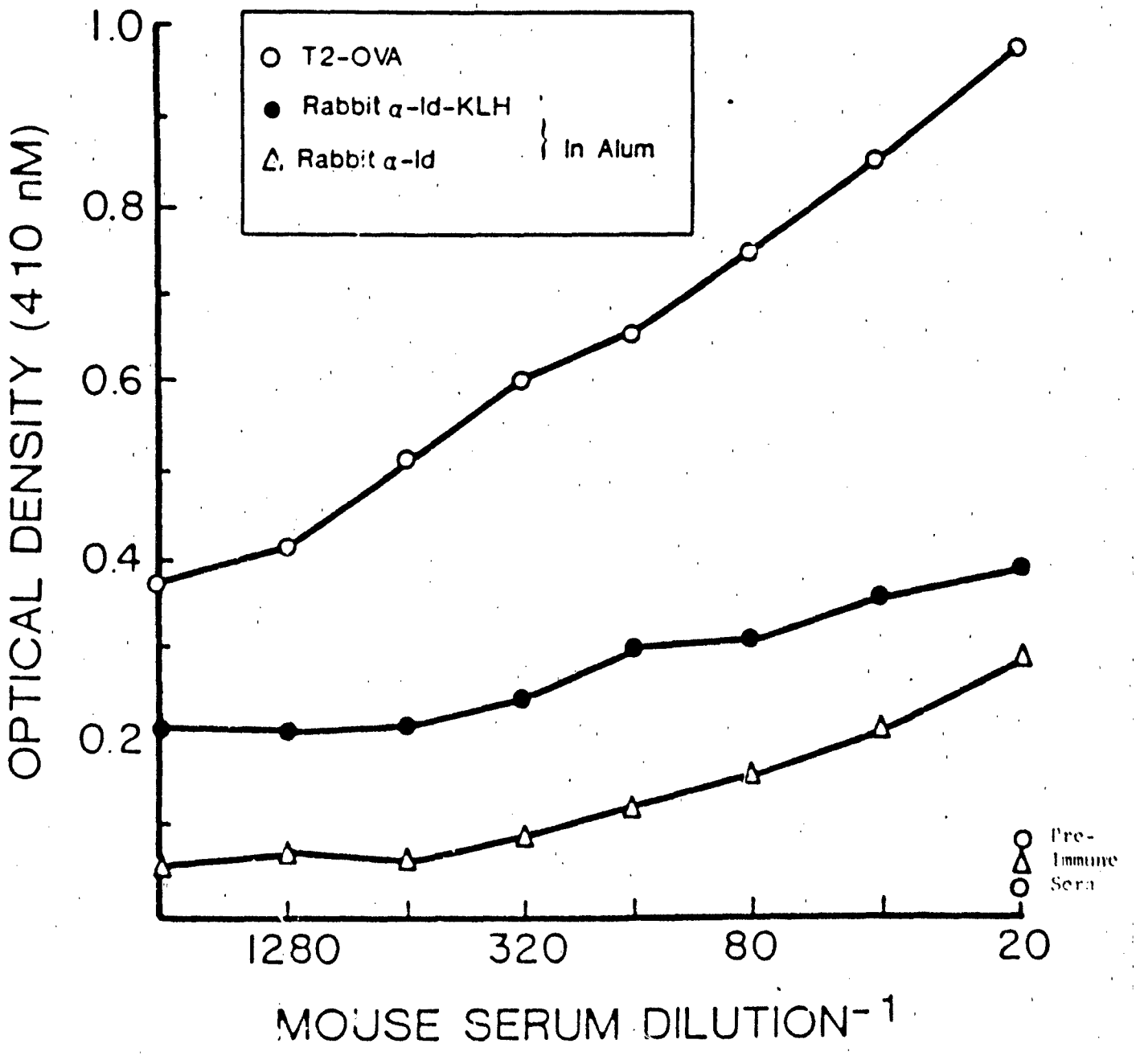


FIGURE 4

Rabbit Anti-Idiotype Induces an Idiotype-Positive Anti-T-2 Response

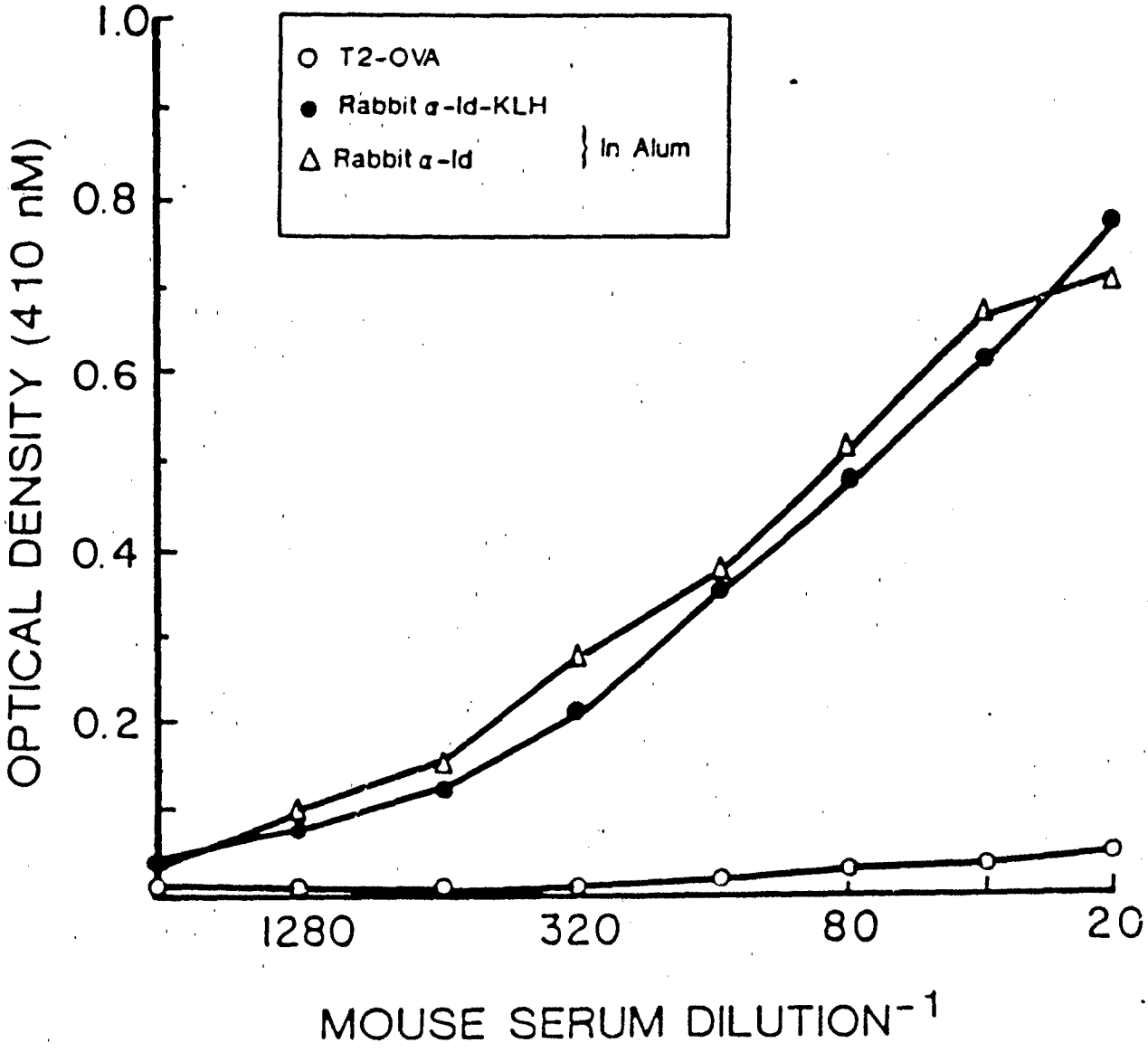
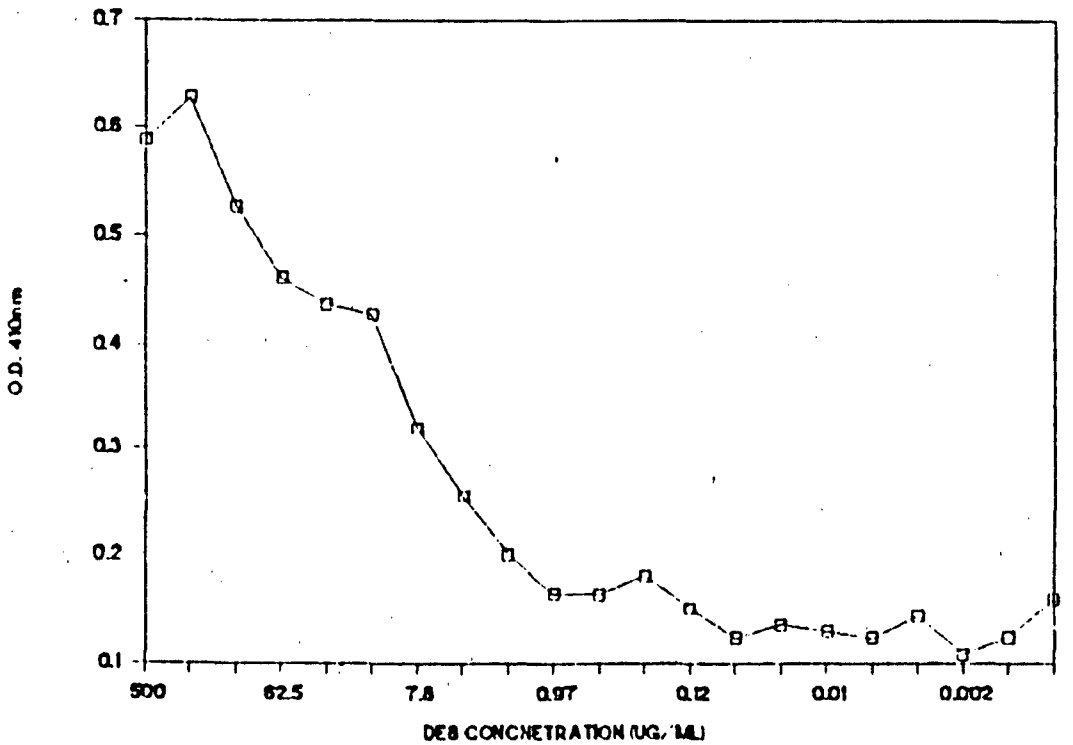


FIGURE 5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0.5
0.4
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0.2
0.1
O.D. 410nm
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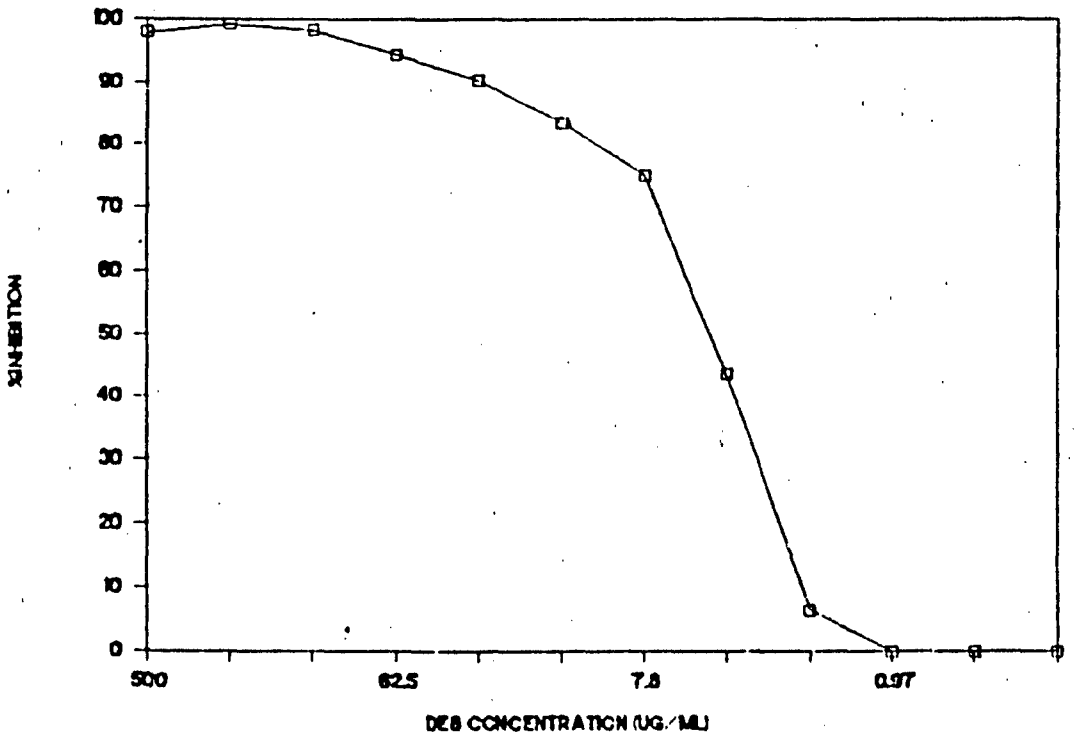
62.5
7.8
0.97
0.12
0.01
0.002
DES CONCNETRATION (UG./ML)

FIGURE 6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80
70
60
50
40
30
20
10
0
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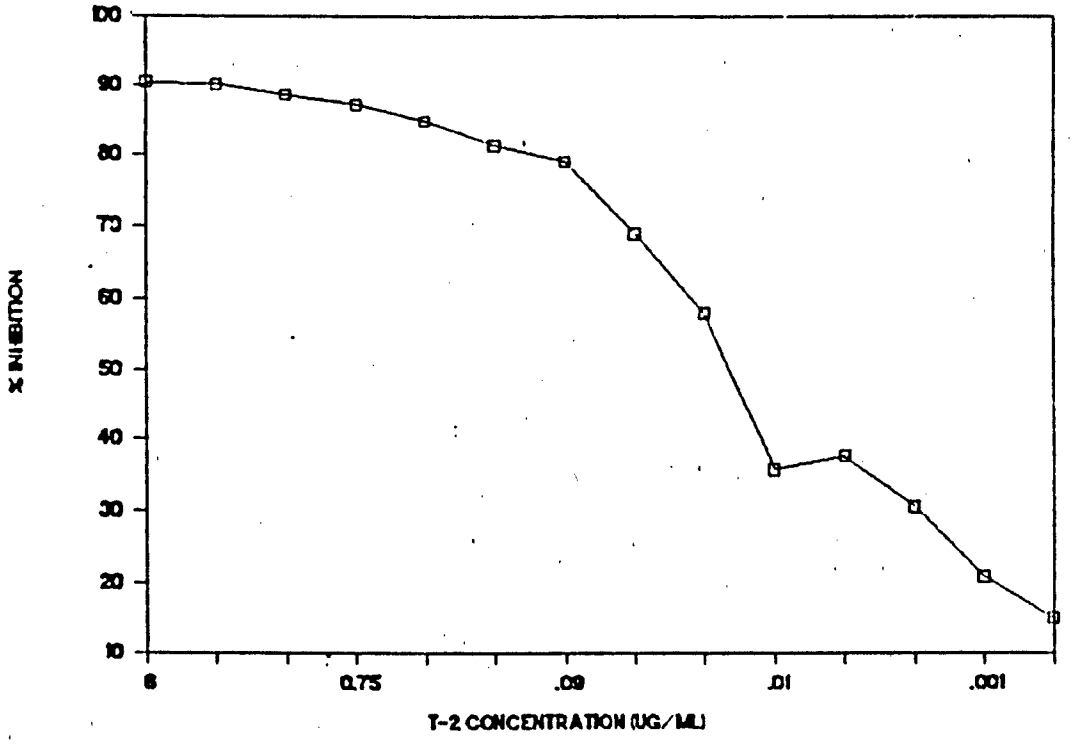
7.8
0.97
DES CONCENTRATION (UG./ML)

FIGURE 7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90
80
70
60
50
40
30
20
10
% INHIBITION
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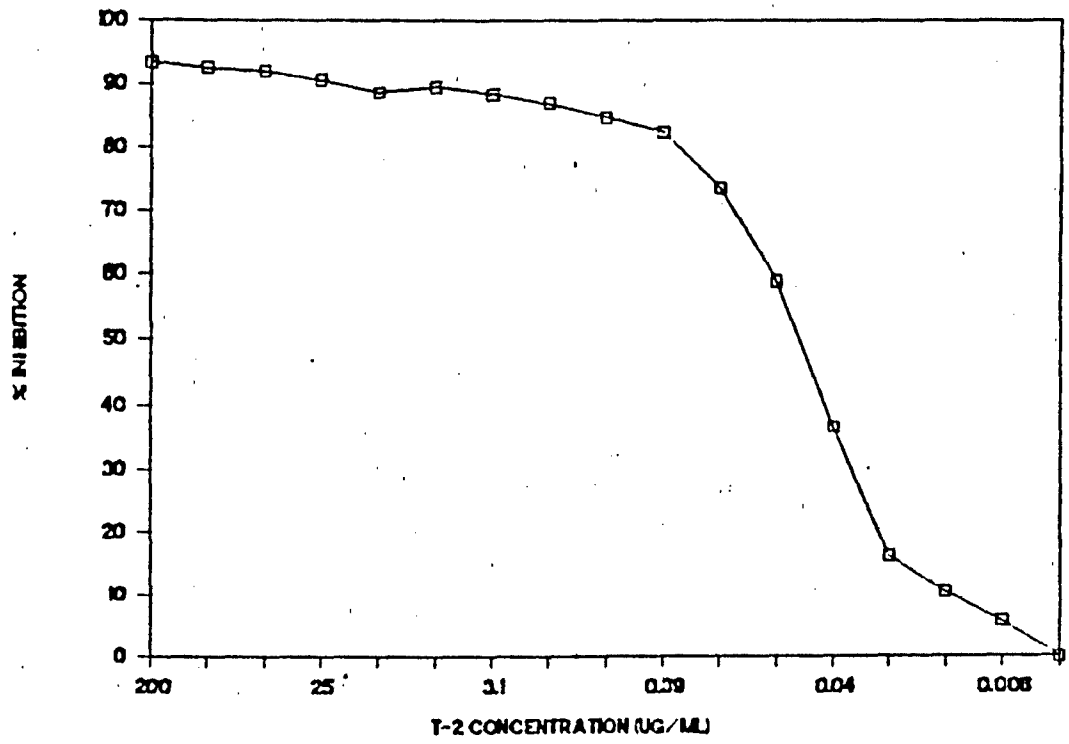
.01
.001
T-2 CONCENTRATION (UG/ML)

FIGURE 8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70
60
50
40
30
20
10
0
% INHIBITION
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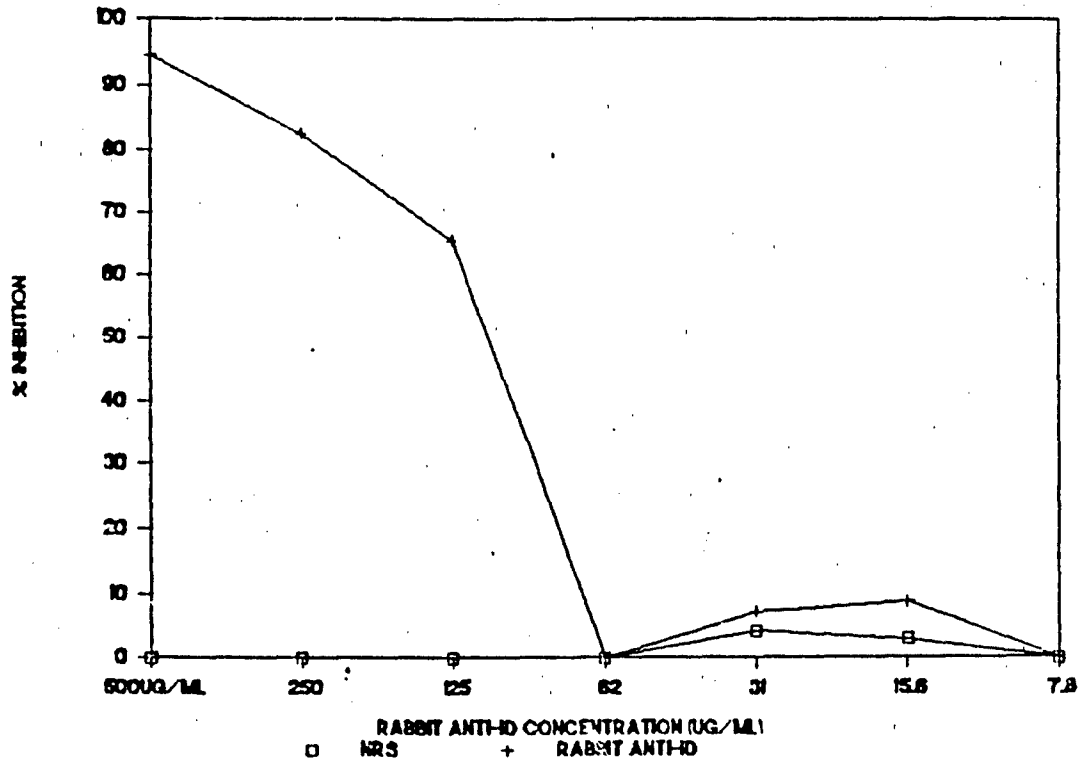
0.005
T-2 CONCENTRATION (UG/ML)

FIGURE 9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60
50
40
30
20
10
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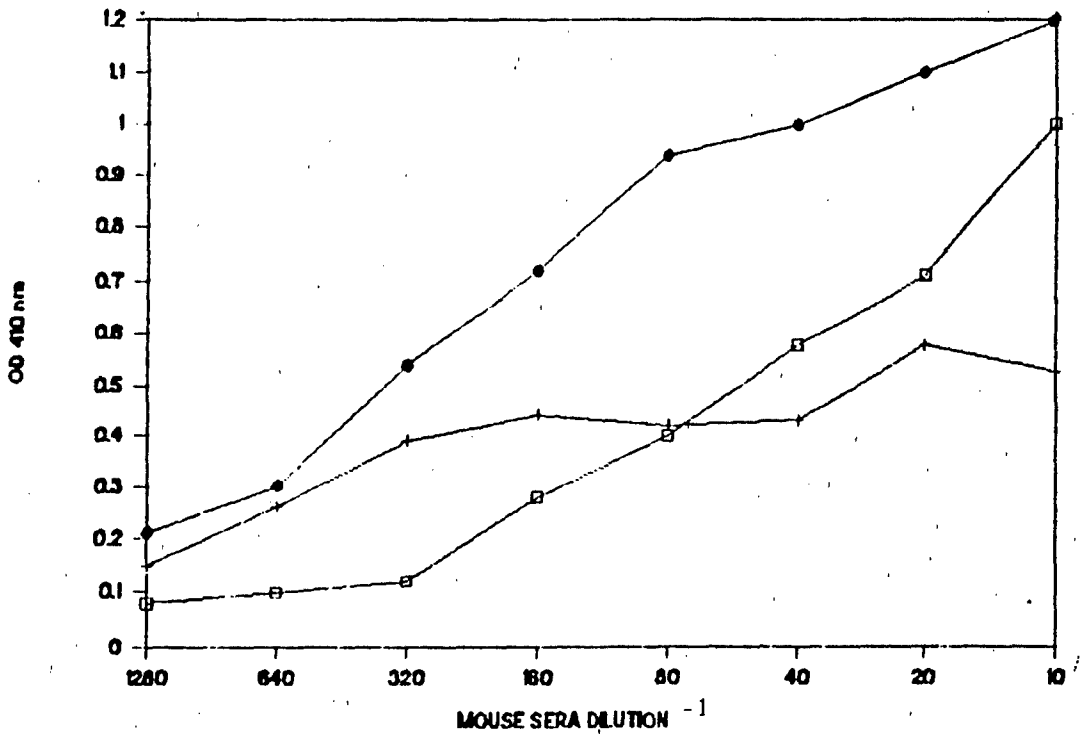
500UG/ML
250
125
62
31
15.6
7.8
RABBIT ANTI-ID CONCENTRATION (UG/ML)
NRS
+
RABBIT ANTI-ID

FIGURE 10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0.9
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0.4
0.3
0.2
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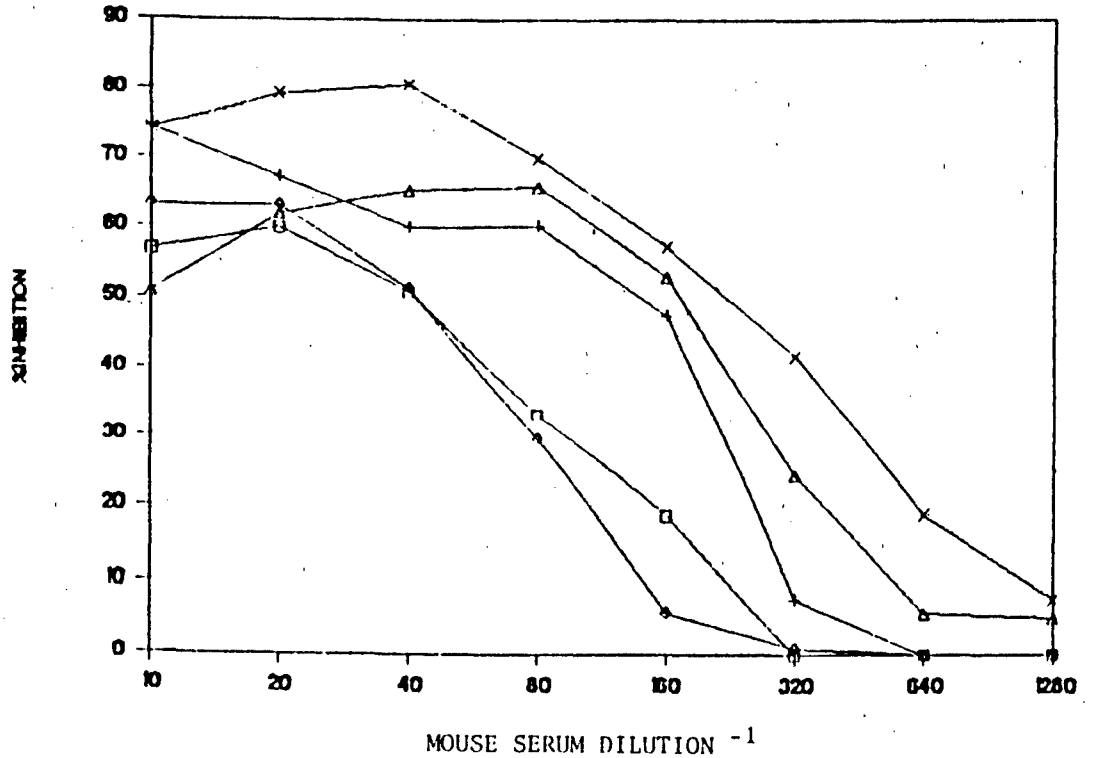
OD 410 nm
1280
640
320
160
80
40
20
10
MOUSE SERA DILUTION -1

FIGURE 11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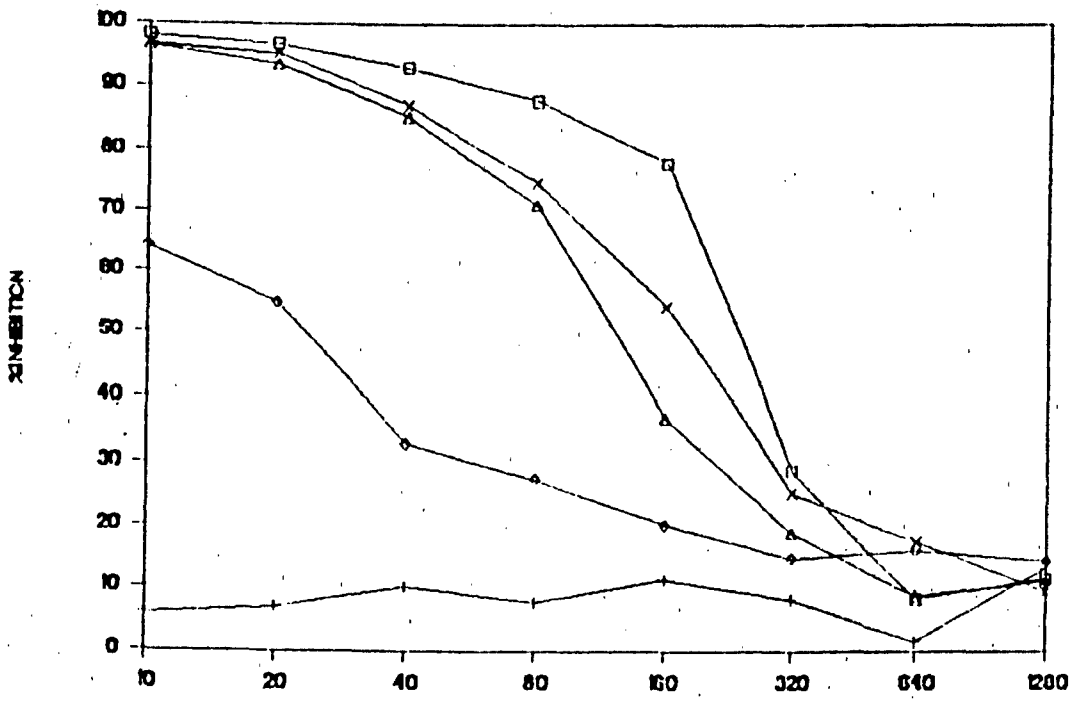
320
640
1280
MOUSE SERUM DILUTION -1

FIGURE 12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1280
MOUSE SERUM DILUTION -1

FIGURE 13

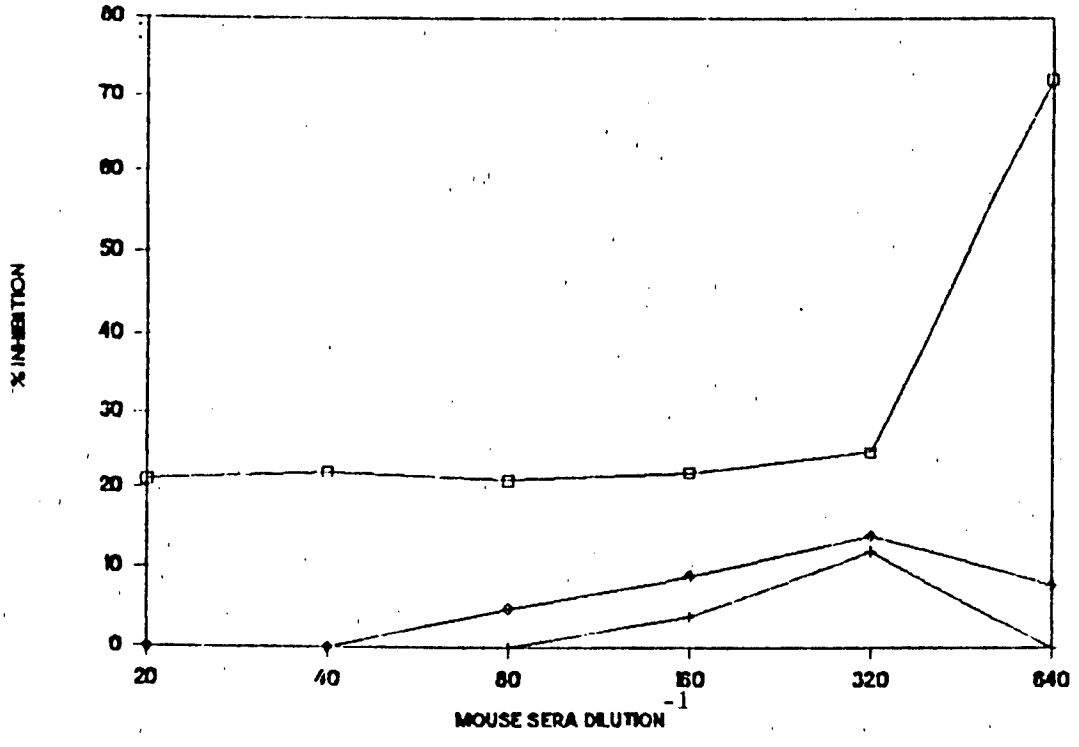
% INHIBITION
80
70
60
50
40
30
20
10
0
20
40
80
160
320
640
MOUSE SERA DILUTION -1

FIGURE 14

Publications:

Chanh, T.C., Reed, R.C., Frenzel, G., and Huot, R.I. Anti-idiotypic antibodies against monoclonal antibody specific for the trichothecene mycotoxin T-2. Fed. Am. Soc. Exp. Biol. 2(4):A677, 1988.

Huot, R.I., Castro, J.A., and Chanh, T.C. Monoclonal antibodies specifically inhibit binding of sodium channel blockers to rat brain membranes. Fed. Am. Soc. Exp. Biol. 2(4):A678, 1988.

Publications Submitted:

Chanh, T.C., Reed, E.P., Huot, R.I., Schick, M.R., and Hewetson, J.H. Anti-idiotypic antibodies against a monoclonal antibody specific for the trichothecene mycotoxin T-2. Submitted.

Huot, R.I., Armstrong, D.L., Hewetson, J.H., and Chanh, T.C. Monoclonal antibodies inhibit the sodium channel blocker tetrodotoxin *in vitro* and *in situ*. Submitted.

Huot, R.I., Castro, J.A., Armstrong, D.L., and Chanh, T.C. Polyclonal rabbit anti-idiotypic antibodies against a protective monoclonal antibody specific for the sodium channel blocker tetrodotoxin. Sixth Ann. Texas Immunol. Conf., Galveston, Texas, submitted.

Schick, M., Hewetson, J., Nabers, P., and Chanh, T. Monoclonal anti-idiotype antibody as vaccine against mycotoxin (T-2)-induced cytotoxicity. Fed. Am. Soc. Exp. Biol., submitted.

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